instruments which, although they increase specificity, reduce overall sensitivity but paradoxically allow increased sensitivity of measurement by increasing the signal : noise ratio. GC–HRMS has successfully been used for the measurement of a calcitriol analogue, hexafluorocalcitriol, with a minimum detectable limit of 2 pg mL^{-1}, which gives this assay the sensitivity to measure plasma calcitriol itself, which circulates at concentrations around 30 pg mL^{-1}. This principle is, of course, generally applicable and most steroids can be detected at lower concentrations by the use of GC–HRMS. This technique has been used mainly by laboratories interested in the detection of anabolic steroids in athletes' urine (e.g. metandienone, stanozolol and clostebol) as a means of detection of drug abuse in sport but also as a means of detecting illicit steroid administration to cattle (4-chlorotestosterone). It has occasionally been suggested, as in the case of nandrolone, that metabolites observed have arisen *de novo* by *in vivo* metabolism from other steroids rather than from exogenous sources. Use of GC–combustion-MS (isotope ratio mass spectrometry) has been shown, by measuring the $^{12}C : {}^{13}C$ ratios, to have considerable potential as a means of distinguishing between exogenous and endogenous sources. **Figure 8** gives a further example of the sensitivity of GC–HRMS which was used to demonstrate the presence of 1α,24-dihydroxyvitamin D_2 in human plasma by focusing on three specific ions and demonstrating that they had a retention time the same as the standard and were present in the same ratio and as they were in the MS of the pure standard. Similar studies with low-resolution MS detection were unable to demonstrate the presence of this steroid.

Conclusion

GC–FID of steroids is today primarily confined to the analysis of urinary steroid profiles, a technique introduced in the 1980s but, as a brief examination of the recent literature will show, still produces valuable clinical information today. Much improved data are obtained when the GC is interfaced with the a mass spectrometer, allowing greater sensitivity and specificity of detection with the added benefit of structural information about unknown steroids. It is interesting to note that C_{21} steroids are usually analysed by immunoassay or LC–MS whereas GC–MS is still widely used for the specific analysis of oestrogens and androgens, particularly in the sports area where the definitive detection of anabolic steroids is required. GC–MS, particularly when high-resolution MS is used, is still more sensitive than LC–MS for steroid assay and EI(+) ionization methodology provides more useful structural information than can be achieved with LC–MS or even LC–MS–MS. It will be interesting to see whether GC–MS will hold its own against LC–MS over the next ten years.

See also: **II/Chromatography: Gas:** Derivatization; Detectors: Mass Spectrometry; High Temperature Gas Chromatography. **III/Steroids:** Liquid Chromatography and Thin-Layer (Planar) Chromatography; Supercritical Fluid Chromatography.

Further Reading

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Liquid Chromatography and Thin-Layer (Planar) Chromatography

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Introduction

This review aims to summarize the application of liquid chromatography (LC) in all its forms, including thin-layer chromatography (TLC), for the analysis of steroids. As LC relies on either adsorption or partition, extraction of the analyte from the matrix, a similar process, has been considered, as has the necessary final step of LC-quantitation. Readers who seek further information are encouraged to use the texts given in the Further Reading section, which are valuable sources of information from which original research references can be obtained as well as information about alternative means of steroid analysis.

Steroids comprise a large group of compounds which occur naturally in both plants and animals. Their structures are all based upon the cyclopentanoperhydrophenanthrene nucleus and all the naturally occurring steroid hormones are synthesized in humans *in vivo* from cholesterol. Some steroid hormones – those derived from vitamin D_3 which are derived from cholesterol precursors – have a broken B-ring and are described as secosteroids. Various chemical modifications of the nucleus can be made by increasing the size of the rings or modifying them in some way to produce large numbers of synthetic steroids. As an illustration of the wide variety of steroids which are available today, the *Dictionary of Steroids* lists around 10 000 compounds. Steroids have a wide spectrum of therapeutic uses and this has encouraged the synthesis of large numbers of synthetic steroids in an attempt to enhance or depress

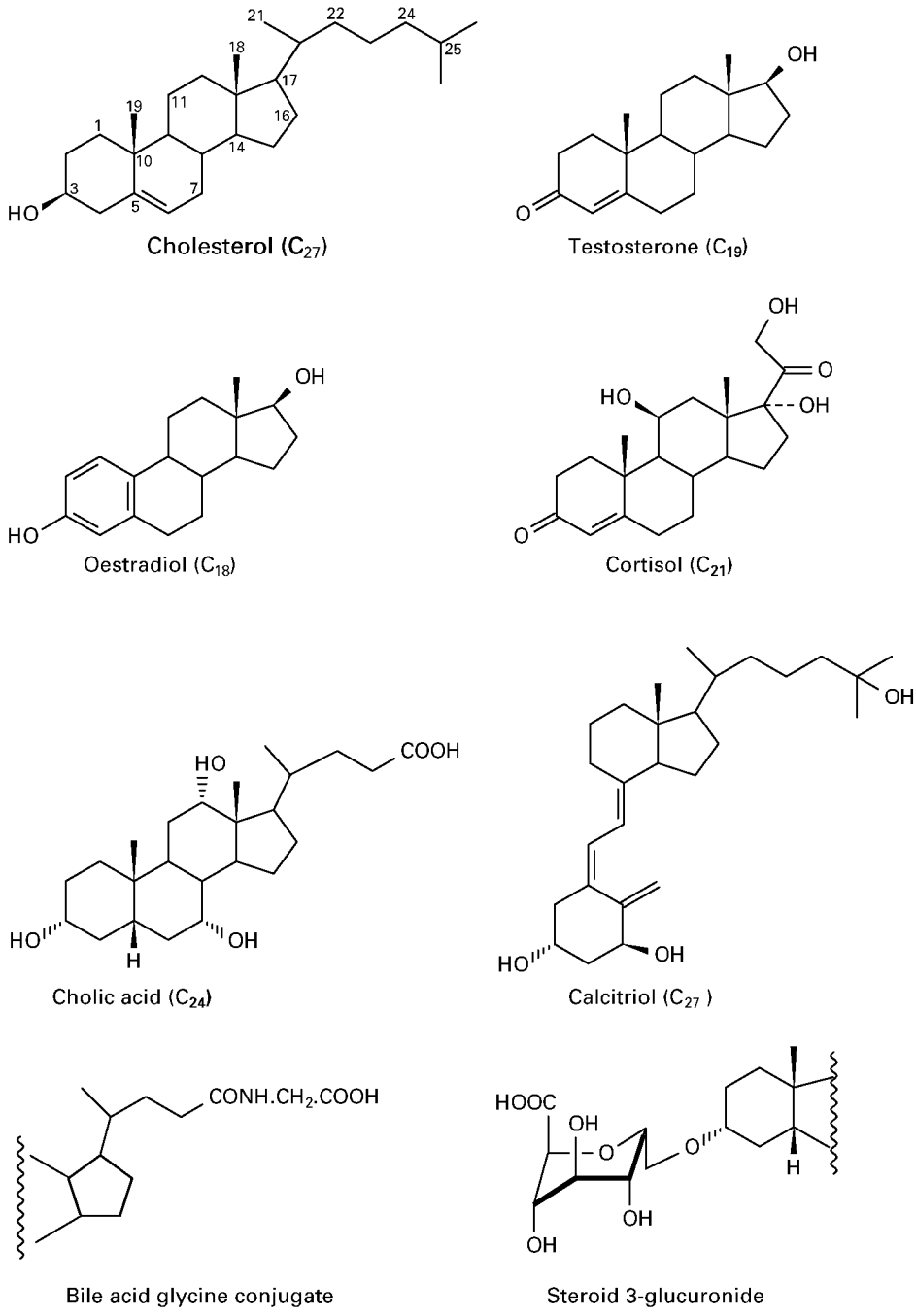

Figure 1 Formulae of some naturally occurring steroids. The numbering system used to identify the individual carbon atoms in the steroid skeleton is also illustrated.

particular physiological responses. From the point of view of a person working in a biomedical environment, the naturally occurring steroids are of particular interest and these include the gluco- and mineralo-corticoids secreted by the adrenal cortex, the sex hormones produced in the gonads, progesterone synthesized in the placenta and the bile acids which aid the digestion of fats. The parent compound of all these naturally occurring human steroids, cholesterol, is an integral part of the structure of cell membranes. The nomenclature of steroids is complicated by the fact that trivial names of many important steroids (see, for example, cortisol and testosterone in **Figure 1**) are still widely used. There are agreed IUPAC rules for the nomenclature of steroids but application of these rules gives rise to long and cumbersome names. Readers who are unfamiliar with steroid nomenclature are referred to the *Dictionary of Steroids* which contains a very useful summary. Figure 1 also illustrates the numbering of some of the important carbons in the steroid nucleus.

It has been estimated that, of the armoury of therapeutic drugs available for prescription in the UK, around 25% either are or contain steroids in their formulation. Because of the physiological and therapeutic importance of steroids and the huge number of different steroids which one may encounter, they represent a considerable analytical challenge. In this short summary of the liquid chromatographic methods for the separation of steroids, attention will be devoted in the main to the separation and quantitation of the naturally occurring steroid hormones and bile acids. Figure 1 gives the structures of some of the important steroids which are found in human serum and examples of their conjugates. Readers who wish to learn more about the infinite variety of steroids are referred to the classic organic chemistry text by Fieser and Fieser and the *Dictionary of Steroids*. A text on the Biochemistry of Steroid Hormones is given in the section on Further Reading.

Steroids are in the main hydrophobic, a property conferred by the nucleus, and this hydrophobicity is modified by hydroxyls and oxo groups on the periphery of the nucleus. Steroids are often conjugated with glucuronic and sulfuric acids, particularly through the hydroxyl at carbon 3. These conjugates are of course more water-soluble than the unconjugated steroid. The side chain attached to carbon 17 of the nucleus in cholesterol contains a further 10 carbons and this side chain is *in vivo* enzymatically cleaved between C_{24} and C_{25} to produce the C_{24} bile acids and between C_{20} and C_{22} to produce steroid hormones. The C_{24} carboxyl can also be conjugated with glycine and taurine which again increase the water-solubility of these molecules. There is therefore quite a wide variation in hydrophobicity between different classes of steroids and within these classes, which can be further modified by conjugation. Most steroids are neutral but the phenolic A ring of the oestrogens and the C_{24}-carboxyl in the bile acids render them acidic and this property can be used for the differential extraction of these two classes of steroids.

In any analytical system there are three interdependent steps: extraction (removal of the analyte from the matrix), separation of the analyte from other compounds, which may interfere in the final step – quantitation. Separation and quantitation are clearly very closely linked in that a quantitation procedure of high specificity may well not require such intensive separation as would be required with a low specificity quantitation. Because of the chemical similarity of the many steroids with each other and, in general, the lack of highly specific quantitation procedures, separation of steroids prior to quantitation is still extremely important. Each of these three stages will be dealt with individually but it must be remembered that, when an analytical procedure is being put together, one stage cannot be viewed in isolation from the others.

Extraction

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Unconjugated steroids are hydrophobic and are relatively easy to extract from the aqueous matrices in which they are often found. The apparent dichotomy of hydrophobicity and the presence of unconjugated steroid hormones in human plasma is resolved when one recognizes the presence of specific binding globulins. The main glucocorticoid, cortisol, has a specific binding globulin (transcortin) and the sex hormones also have a specific globulin which transport these steroids in human blood. To extract steroids therefore from serum or plasma, it is necessary to disrupt the steroid–protein binding. Some steroids, such as cholesterol or vitamin D_3, are particularly difficult to extract and it is thought that this occurs because they become involved in lipoprotein structure. It is possible to overcome this difficulty by extracting with ethanol–ammonium sulfate or pentylamine. Most steroids however can be extracted from plasma/serum or incubation medium with a simple Bligh & Dyer extraction which utilizes methanol–chloroform (2 : 1, v/v). A simple wash of the organic extract with alkaline buffer will remove fatty acids which are also extracted but may interfere in subsequent analysis. However, washing with alkaline buffer may also remove substantial quantities of acidic steroids such as bile acids and oestrogens. In the past, ether was a common solvent for steroid extraction as it is less dense than water and the

aqueous layer can be frozen with solid CO_2 and the organic extract poured off. However, in more safety-conscious times, the flammability of ether has reduced its use.

The extraction of steroids using solvents is discussed in more detail in texts cited in the Further Reading section. Such procedures should not be viewed solely as a means of extraction as judicious choice of solvents can give a surprising degree of selectivity and particular steroid groups can be preferentially extracted. Steroid conjugates, which are more difficult to remove from aqueous media, can also be extracted from, for example, human urine using ether–isopropanol after saturation of the urine with ammonium sulfate–so-called forced extraction. The conjugates can then be hydrolysed using enzymes (β-glucuronidase or sulfatase) or, in the case of sulfate, acid solvolysis can be utilized. The need for hydrolysis of steroid conjugates depends upon the subsequent separation and quantitation techniques. Clearly hydrolysis of conjugates loses information which may or may not be of importance. As will be seen later, modern methods of analysis using LC–mass spectrometry (LC-MS) allow for the separation and quantitation of intact conjugates and it may therefore be unnecessary to hydrolyse before proceeding to the separation or steps.

Solvent extraction leads to the generation of relatively large volumes of potentially hazardous solvents which need to be removed, usually using a rotary evaporator or simply blowing nitrogen onto the solvent while heating it not higher than about 40°C. Solvents which have high boiling points or solvent mixtures containing water are particularly difficult to remove. Because of these problems, other methods of extraction have been investigated and, in the case of steroids, major advances have been made, particularly in the field of solid-phase and immunoaffinity extraction. As examples of solid-phase extraction (SPE), one can consider the use of microparticulate silica for the extraction of steroids and vitamin D metabolites. There are a wide variety of such materials which are all based upon microparticulate silica, modified by derivatizing the polar groups with silanes (i.e. octadecylsilane (ODS) C_{18}, is widely used). Structures and performance of the solid-phase materials can most readily be obtained by looking at the catalogues of manufacturers of these materials. In the UK a very useful source of information is the catalogue of International Sorbent Technology, a major supplier of such materials (e.g. Bond-Elut). Sep-Pak is another useful proprietary brand, manufactured and marketed by Waters. As an example of the use of these materials, vitamin D_3 metabolites in plasma, although not vitamin D_3 itself, can be extracted with acetonitrile, which disrupts the protein binding. After centrifugation to remove the precipitated protein, the extract is then poured through an ODS-silica column or cartridge (Sep-Pak C_{18} or Bond-Elut C_{18}) and the metabolites of interest can be eluted, after washing, with methanol. A similar procedure can be used for other steroids in plasma or urine and often their conjugates as well.

SPE techniques for steroid extraction, although not specific, are increasingly used in preference to solvent extraction. The SPE material can often be reused many times, if satisfactory washing procedures are applied between each use. Highly specific extraction can be achieved using immunoaffinity columns where antibodies to specific steroids or groups of steroids are immobilized by linking to Sepharose. Aqueous mixtures of steroids can then be passed down the column: steroids of interest are bound to the antibody and after the unwanted steroids have passed through the column the steroid antibody binding can be disrupted and the steroid(s) of interest eluted. In ideal cases using highly specific antibodies and a relatively specific quantitation, it may not be necessary to carry out any further separation procedures. Sometimes simple procedures can be extremely effective. As an example, the binding of some plasma steroids to specific globulins can allow selective extraction as ammonium sulfate can sometimes be used to precipitate the specific globulin, which brings the steroid of interest with it.

Separation

Today high performance liquid chromatography (HPLC) is widely used for steroid separation because this technique can be directly linked to quantitation. This is, however, not to imply that other methods of separation may not find use in particular applications. Open-column chromatography (either adsorption or partition) is still used with advantage on occasions. A major and very useful separatory technique is TLC and, if microparticulate material is used, it becomes high performance TLC (HPTLC). TLC is particularly advantageous in that numbers of separations can be carried out at the same time and the apparatus required is inexpensive. For these reasons and because TLC is relatively easy to carry out, it is still quite widely used and scrutiny of recent papers on steroid separation confirms this. It is however true to say that very little development of TLC systems has occurred in the last 20 years and most systems are based upon methods described prior to 1980. TLC can also be used as a preliminary means of investigating new solvent systems for the separation of steroids by HPLC.

Column Chromatography

This procedure utilizes adsorbent materials such as alumina, Florisil (magnesium silicate) or silica. Steroids are adsorbed to these materials and are eluted by solvents of increasing polarity. The order of elution depends upon the solvent and the differing polarity of the steroids under consideration. Clearly, the more polar a steroid (in general, this means the more hydroxyls it contains), the more hydrophilic the steroid becomes and the longer it takes to be eluted. These adsorbent materials are usually packed into small columns (for example, Pasteur pipettes can be used) and exquisite separations can now be achieved by the use of microparticulate silica. These columns are simple to use, provide rapid separations and have the advantage that after washing they can be reused many times. Classical steroid separations using columns can be achieved by partition chromatography using biphasic solvent systems. The stationary aqueous phase is mixed with celite (a diatomaceous earth) and this material is packed into the column; the steroid mixture is applied and eluted with the organic mobile phase. This is rather a cumbersome procedure but does offer a considerable degree of separation. It is not widely used today, although examples of partition chromatography used in this way can still be found. An example of this technique for the separation of some progesterone metabolites is illustrated in **Figure 2**.

Modified cross-linked dextran columns (i.e. Lipidex) have been used to provide steroid separations and can find more mundane uses such as the removal of excess trimethylsilylimidazole reagent when forming steroid trimethylsilyl ethers. A similar material, Sephadex LH-20, has also been used to fractionate steroids into free steroids, glucuronides, mono- and disulfates. Sephadex can also be modified by linking it to form, for example, diethylaminoethyl-substituted material which can act as an ion exchanger as well as a size exclusion material. These ion exchange/gel filtration columns are particularly useful for the separation of steroid conjugates; for example, bile acids can be separated by the use of another modified Sephadex, PHP-LH20. **Figure 3** shows such a system for the separation of steroids and their conjugates from human urine prior to gas chromatography–MS (GC-MS).

Thin-Layer Chromatography

TLC is in effect very similar to column chromatography and is based on the same principles. A thin layer of adsorbent or inert material is spread on a glass, plastic, or aluminium sheet. For the separation of steroids using organic solvents, the use of thin layers on plastic sheets is not recommended and either glass or aluminium should be used. After separation, steroids may be recovered from the plate by scraping off the thin layer and eluting the steroid of interest.

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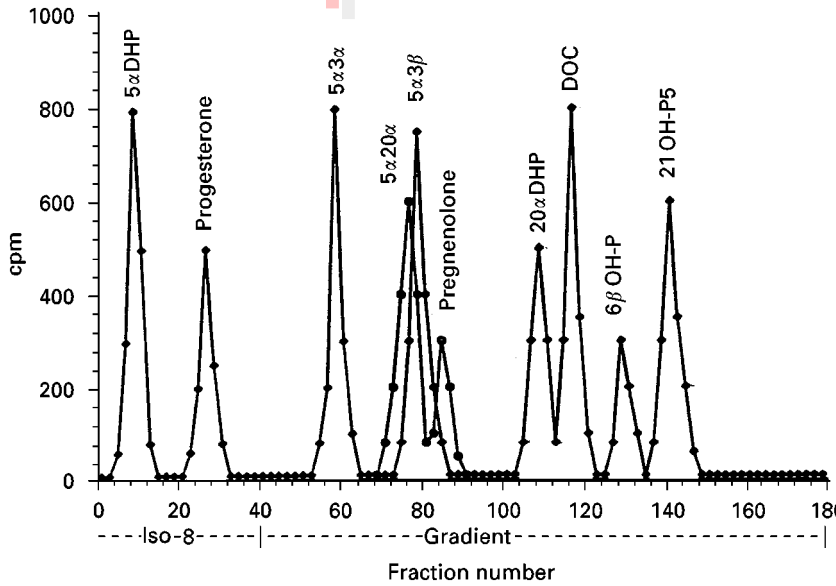

Figure 2 Chromatogram of selected C_{21} steroids in urine. Radiolabelled and nonradiolabelled authentic steroid standards were applied to a 40 g column of celite–propylene glycol and eluted with iso-octane (e.g. Iso-8, 40 fractions) and thence a linear gradient of iso-octane–ethyl acetate (e.g. *Iso*-8, 140 fractions). Steroids are identified as follows: 5αDHP, 5α-pregnane-3,20-dione; 5α3α, 5α-pregnan-3α-ol-20-one; 5α20α, 5α-pregnan-20α-ol-3-one; 5α3β, 5α-pregnan-3β-ol-20-one; 20αDHP, Δ^4-pregnen-20α-ol-3,20-dione; DOC, Δ^4-pregnen-21-ol-3,20-dione; 6βOH-P, Δ^4-pregnen-6β-ol-3,20-dione; 21OH-P5, Δ^5-pregnen-3β,21-diol-20-one. (Reproduced with permission from Dombroski RA, Casey ML and MacDonald PC (1997) *Journal of Steroid Biochemistry* 63: 155–163.)

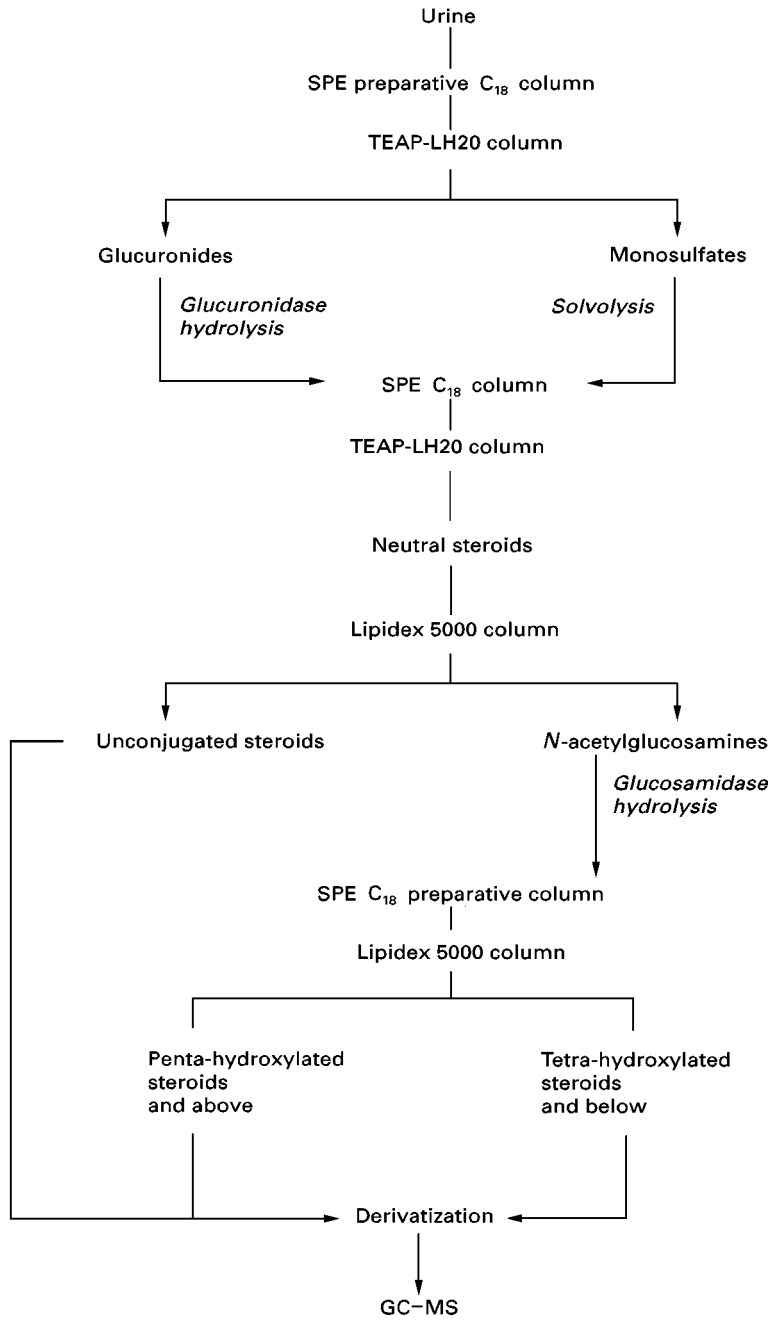

Figure 3 Use of solid-phase extraction and Sephadex-based packing materials for the extraction and separation of steroids and their glucuronide, sulfate and *N*-acetylglucosaminide conjugates from human pregnancy urine prior to analysis by gas chromatography–mass spectrometry (GC-MS). (Reproduced with permission from Meng LJ, Griffiths WJ and Sjövall J (1996) *Journal of Steroid Biochemistry and Molecular Biology* 58: 585–598.)

The advantage of thin layers on aluminium foil is that the area of interest can be cut out and the whole area of the plate can be eluted without removing the adsorbent.

The commonest forms of TLC for the separation of steroids use adsorbent thin layers of either alumina or silica gel, although there are descriptions of reversed-phase partition TLC. Use of microparticulate silica (HPTLC) confers a slightly enhanced selectivity in separation but HPTLC is not widely used. The steroid extract of interest is applied to the bottom of the plate and eluted with various solvents. Because of the ability of such separation systems to deal with a number of separations at once, they are still quite widely used

today. The vast majority of separation systems use silica gel but alumina has been used to separate C_{19} androgens. The resolution is achieved by judicious choice of solvents as the number of adsorbents available is limited.

Very little development of this separation technique for steroids has been carried out over the last 20 years as attention has been diverted towards the development of HPLC. Recent TLC systems using silica gel and eluting with ethyl acetate : petroleum ether have enabled hydroxylated metabolites of progesterone to be resolved and again using silica gel with benzene–heptane–ethyl acetate or chloroform–ethyl acetate has enabled resolution of catechol oestrogens. In many instances, however, suitable solvents can no longer be used as they pose unacceptable risks of flammability or toxicity (e.g. benzene). A study of papers published in the last 10 years involving the use of TLC for the separation of steroid metabolites indicates that most procedures were published many years ago, although the recent use of cyclodextrin in the TLC of bile acids (cyclodextrin in the mobile phase) and steroidal drugs (cyclodextrin polymer-coated silica) has been an interesting innovation.

Table 1 lists some examples of the recent use of TLC for the separation of steroids and clearly illustrates the increasing reliance on silica gel as the adsorbent, using the differing polarity of the elution solvent mixture for resolution. Most of the development systems are modifications of previously published solvent mixtures. Table 1 also illustrates the use of two-dimensional TLC and multiple development in the same direction. It is also possible to utilize one solvent mixture for de-fatting, followed by another to separate the steroids of interest or sequential solvent systems, separating first one group of steroids followed by a second solvent mixture to separate another steroid group.

The problem associated with the use of TLC for the separation of steroids is to locate the position of the steroids after chromatography and some methods

Table 1 Some examples of recent use of TLC for steroid separation

Steroids	*Adsorbent*	*Development solvent*	*Detection*
Side chain cleavage products of ^{14}C-cholesterol	Silica gel G	Di-isopropyl ether–hexane–acetic acid (70 : 30 : 2)	Radioactivity by autoradiography
Metabolites of 7α-OH-androstene-3,17-dione	Silica gel	Ethyl acetate–hexane (3 : 7)	
7-Hydroxylated DHEA	Silica gel F254	Ethyl acetate	UV absorption
Oestrogens	Keiselgel 60 F254	Toulene–acetone (4 : 1)	UV absorption and iodine vapour
^{3}H corticosteroids	Silica gel 60 F254	Choloroform–acetone (23 : 2)	Autoradiography
^{3}H testosterone metabolites	Polygram SIL G	Methylene chloride–diethyl ether (4 : 1)	Autoradiography
Oestrogens	Whatman LK6DF Silica gel 60	Ether–chloroform (6 : 4) Chloroform–ethyl acetate (4 : 1)	Iodine vapour
Corticosteroids	Silica gel	Ethyl acetate–isooctane (7 : 3)	
Corticosteroid sulfates	Silica gel	Chloroform–methanol–NH_4OH (65 : 25 : 0.1) Ethyl acetate–methanol–NH_4OH (75 : 25 : 2)	
Progesterone metabolites	Fisherbrand F254	Two-dimensional TLC $\times$ 2 with chloroform–ether (10 : 3) then $\times$ 2 in hexane–ethyl acetate (5 : 2)	Iodine vapour and UV absorption
Androst-16-ene biosynthesis	PE-SIL-G Silica gel	Chloroform–acetone (9 : 1) and hexane–ethyl acetate (5 : 3.5) Run $\times$ 2	Iodine vapour and UV absorption
Metabolites of ^{3}H-progesterone, -pregnenolone and -DHA	Fisher silica gel 60 F254	Two-dimensional TLC firstly to de-fat in cyclohexane–ethyl acetate (95 : 5) $\times$ 5–7. Then toluene–acetone (8 : 2) $\times$ 2, finally $\times$ 2 in first direction with cyclohexane–ethyl acetate (1 : 1)	Autoradiography

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used for this purpose are also summarized in Table 1. For steroids containing UV-absorbing groups, such as the δ-4-3-oxo group in the A-ring of most active steroid hormones and the aromatic ring in the oestrogens, visualization can be achieved by examining the plate under UV light. To improve the detection of steroids absorbing at around 240 nm, most commercially available TLC plates have a fluorescent material incorporated which improves detection of the absorption of UV light at around 254 nm. Other techniques are often destructive and require reagents to be sprayed on to the plate. Thus, to avoid destroying the steroid of interest, it is necessary to have standards run on the same plate at the side so that the position of the steroids of interest can be gauged. There are other nondestructive means of visualization, such as the use of iodine vapour but these are not always satisfactory, particularly at low concentrations. One advantage of TLC is in the separation of radiolabelled steroids which can be visualized by autoradiography.

High Performance Liquid Chromatography

The application of HPLC to the separation of steroids has been extensively studied over the last 20 years. A detailed and comprehensive review of the HPLC of steroid hormones was published in 1988 and updated in 1995 (see Further Reading). HPLC is in essence no different to the column and thin-layer systems discussed above, although the resolving power of modern HPLC columns is significantly greater: some reversed-phase columns achieving 60 000–80 000 theoretical plates per metre. New solvent systems for normal-phase HPLC can be investigated using TLC. Columns, because of the high resolving power which can now be achieved, are usually quite short (100–300 mm long with an internal diameter of 4.6 mm). Microbore columns (<2 mm in diameter) can be used to reduce mobile phase consumption but may present practical problems because of the limitation in sample capacity. However, microbore columns may have considerable application in LC-MS, because of the low solvent flow rates. Silica contains free OH groups and these can be modified, replacing the OH for example with cyanopropyl (CN, used for the separation of corticosteroids) or aminopropyl (NH_2, used for the separation of oestrogens). Improved resolution of particular steroids can sometimes be achieved using these modified silicas and, for example, silica-CN gives selective retention of steroids containing oxo groups, and has been particularly useful in the separation of the 25-hydroxyvitamin D_3-23,26-lactone which is difficult to resolve from 24,25-dihydroxyvitamin D_3 in conventional normal-phase HPLC systems. Reversed-phase HPLC is usually based on silica modified with silanes of various chain lengths – C_{18} (ODS) silica is the most widely used material for this purpose. Column packings based on synthetic material are now being made available and may in the future replace silica. Considerable advances in the production and quality of these packing materials have been made over the past 10 years and thus reproducibility of separations has improved.

Most steroid separations today use reversed-phase systems with C_{18} or C_8 silica, although an exception to this general rule is the separation of metabolites of vitamin D which use normal-phase systems eluting with hexane–isopropanol–methanol or hexane–methanol–chloroform. Binary hexane–isopropanol systems give significant tailing and resolution problems which are improved by the use of ternary solvent mixtures. These normal-phase systems give excellent resolution of the metabolites of vitamins D_2 and D_3 but do not separate the vitamins themselves (**Figure 4**), which can be achieved using reversed-phase ODS–silica eluting with methanol–water. If steroids are to be recovered from the eluting solvent, it is clearly advantageous if normal-phase systems with sufficient resolving power can be developed as the removal of aqueous solvents used in

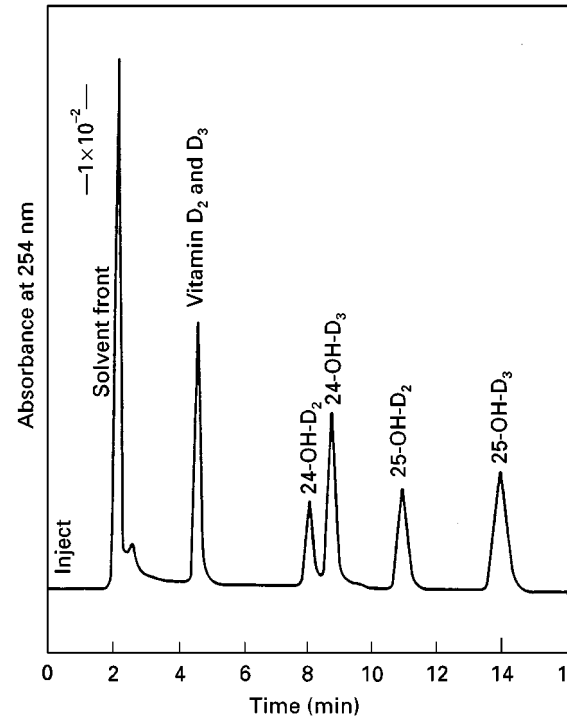

Figure 4 LC separation of vitamins D_2 and D_3, 24-OH-D_2, 24-OH-D_3, 25-OH-D_2 and 25-OH-D_3, using Zorbax-SIL and eluting with 2.5% isopropanol in hexane. (Reproduced with permission from Jones G and DeLuca HF (1975) *Journal of Lipid Research* 16: 448–453. Copyright 1975 FASEB.)

Table 2 Examples of some recent HPLC systems for the separation of steroid hormones and bile acids

Steroid group	*Carbon atoms*	*Stationary phase*	*Mobile phase*	*Comments*
Vatamin D	C_{27} (D_3 series)	Zorbax ODS	MeOH (MeCN)–H_2O (acid)	Separation of D_3 and D_2
Secosteroids	C_{28} (D_2 series)	Zorbax CN	Hx–IPA–MeOH	Retards metabolites containing oxo groups
		Zorbax SIL	Hx–IPA–MeOH	Usual system for metabolite resolution
Bile acids	C_{24}	YMC GEL C8	MeCN–H_2O + cyclodextrin	Bile acids and their conjugates as bromopyrenacyl esters
		Bile Pak II	Gradient with MeCN–MeOH–PO_4 buffer	Bile acid conjugates using post-column immobilized 3αOHSDH and fluorescence detection
Corticosteroids	C_{21}	Cosmosil C_{18}	MeOH–H_2O gradients	Corticosterone and DOC
		Novapak C_{18}	MeOH–buffer	Electrospray–MS of DHE and DHF
		Nucleosil C_8	MeCN–H_2O + HCOOH	Dexamethasone metabolites
Progesterone metabolites	C_{21}	Finepak SIL-C_{18}	Tetrahydrofuran or MeCN–imidazole buffer	Pregnane- and pregnene-diols
Androgens	C_{19}	NovaPak C_{18}	Gradient of MecN–H_2O	DHEA and 7-OH-DHA in newborn foal's blood
		Hypersil BDS-C_8	Gradient of 7.5 mmol L^{-1} NH_4Ac–MeOH	LC-MS of testo. and epi-testo. glucuronides/sulfates
		Hibar Lichrosorb-DIOL	Hx–IPA	Metabolites of DHT and androstanediol
Oestrogens	C_{18}	Wakosil C_{18}	MeOH–H_2O	Plasma oestrogens–pre-column derivatization as benzimidazoles
		Beckman ODS	MeCN–H_2O + cyclodextrin	Urinary oestrogens

reversed-phase separations causes considerable difficulties. Corticosteroids have been successfully separated using normal-phase systems based on DIOL-silica sorbents (-Si-2,3-dihydroxypropoxypropyl) and ion exchange HPLC has also been used for the separation of polar oestrogens. Some recent examples of typical HPLC systems used for the separation of steroids are given in **Table 2**, which illustrates the fact that most methods in use today are reversed-phase systems using ODS/C_{18} packings.

All steroids are susceptible to permanent absorption and/or chemical destruction or modification by unsilanized glass surfaces, exposed metal and by non-inert supports and, for quantitative HPLC, great care must be taken to remove or reduce such materials. Sorbents containing accessible hydroxyl groups should not be used for the separation of 18-hydroxylated steroids as chemical modification of these steroids may occur during chromatography. Although not relevant to HPLC, it should be pointed out that partially end-capped ODS–silica can still be used with advantage in particular situations for rapid separations after SPE. As an example, the use of Bond-Elut C_{18}-OH allowed both extraction and subsequent separation on the same cartridge in a method for the assay of calcitriol (1,25-dihydroxyvitamin D_3).

One particular advantage of HPLC is that it is not destructive and can thus be used for the separation and quantitation of intact steroid conjugates. These polar molecules are not susceptible to analysis by GC as the high temperature necessary to maintain steroids in the vapour phase causes hydrolysis of the conjugate. Examples of the application of HPLC to the separation of androgen and oestrogen glucuronides and the application of LC-MS(MS) to the separation of intact conjugates and steroid fatty acid esters are given in the Further Reading section. An example of such a separation is given in **Figure 5**. Until the advent of HPLC, steroid conjugates had to be hydrolysed prior to resolution and important information was thus lost. The use of LC, particularly when coupled to MS(MS), has allowed resolution and quantitation of intact conjugates together with structural information. HPLC systems for steroid conjugates are usually but not exclusively reversed-phase primarily based on ODS-silica eluting with methanol–, tetrahydrofuran–, acetonitrile–water or buffer solvent systems. The conjugates can be detected in the same way as nonconjugated steroids and this is discussed below.

Detection/Quantitation

This is the final and perhaps most important step and there are a variety of methods which can be used for the detection or quantitation of steroids. These methods are usually considered only in conjunction with HPLC as they are usually insufficiently specific

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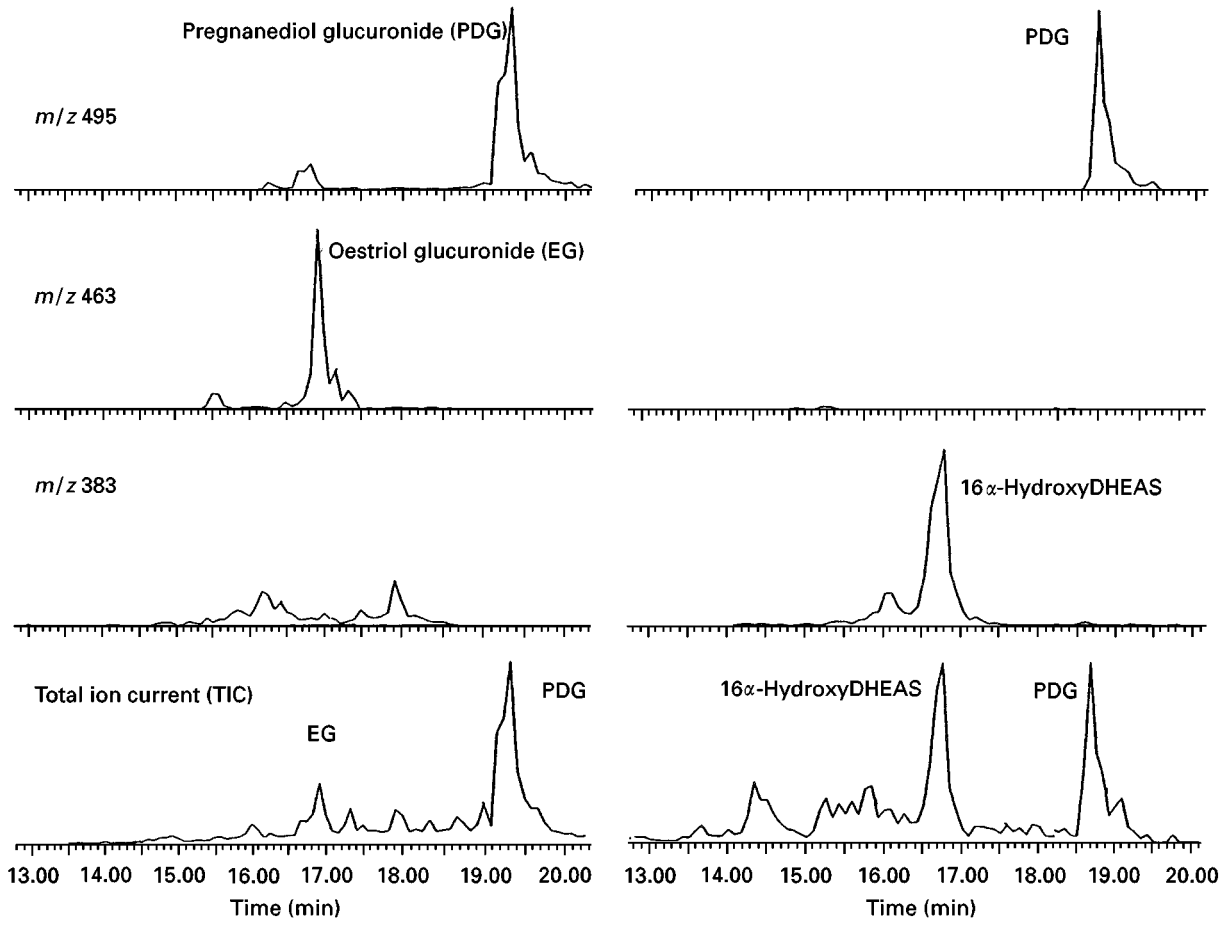
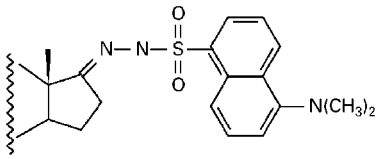


Figure 5 Analysis of maternal urine for the detection of placental sulfatase deficiency (PSD). The microbore HPLC–ES–MS chromatograms represent selected ions for detection of pregnanediol glucuronide, oestriol glucuronide and 16α-hydroxy-DHEA sulfate. The selected-ion chromatograms on the left of the figure are from a normal individual and those on the right are from a patient with PSD. Oestriol and its glucuronide cannot be synthesized by women with PSD, and the precursor 16α-hydroxy-DHEA sulfate is a dominant steroid in urine. The amount injected into the microbore column was equivalent to 25 μL of urine, the eluate being split 10 : 1 prior to the mass spectrometer. The column used was a Reliasil number 9, 1 × 100 mm. Solvent A was 98% 10 mmol L^{-1} ammonium acetate in H_2O, 2% acetonitrile; solvent B was 100% acetonitrile. The gradient was 2% B to 30% over 20 min, and a flow rate of 40 μL min^{-1} was used. From Makin HLJ, Gower DB and Kirk DN (eds) *Steroid Analysis* (1995) Reproduced with permission from Kluwer Academic Publishers.

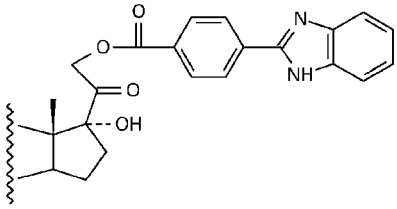
to be used without rigorous prior separation of interfering steroids – the exception to this being immunoassay which can, depending upon the antibody, be sufficiently selective and sensitive for use directly on plasma/serum without extraction or prior purification. There are a number of such assays available today but their uncritical use can lead to problems. An example of this is an immunoassay for 17-hydroxyprogesterone, developed for use with adults. This was applied to the diagnosis of a genetic defect in cortisol biosynthesis, congenital adrenal hyperplasia. The possible interference in this assay by 17-hydroxypregnenolone sulfate which is normally produced in very young children was not appreciated. A simple solvent extraction procedure overcame this problem once it was detected.

It is often the case that one or other of the simple purification procedures, such as selective solvent extraction, use of mini-columns or even TLC on small plates can greatly enhance specificity and there are many examples of this in the literature. It is often unnecessary to use expensive HPLC systems to resolve these steroid analytical problems and consideration of the physicochemical properties of the steroid of interest may often suggest a simple non-HPLC solution. Immunoassay is however one of the most sensitive methods of steroid quantitation and, coupled with HPLC, even with a relatively non-specific antibody, can provide a system with both high specificity and sensitivity. It does however require collection of the eluent – so called offline detection.

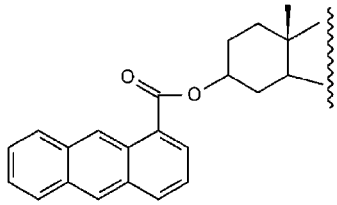
1. Dansyl hydrazones of 17-oxosteroids



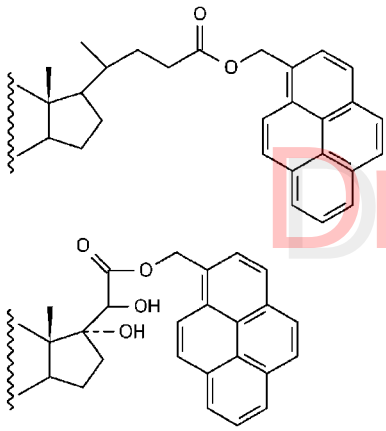
2. 2(4-carboxyphenyl)-5,6-dimethylbenzimidazole esters of cortoic acids

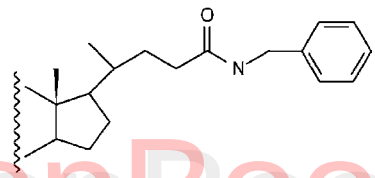


3. 3(1-anthroyl) and 1-pyrenylmethyl esters of bile acids and cortoic acids



4. N-benzylamides of bile acids





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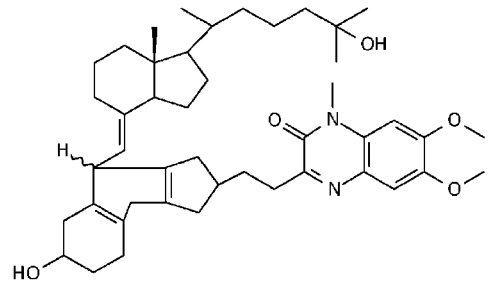
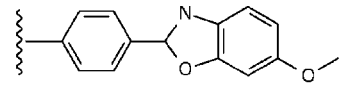
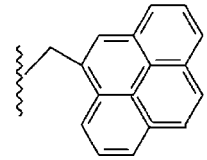
5. Substituted 1,2,4-triazoline-3,5-3,5-dione (TAD) adducts of vitamin D metabolites



2(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl)-ethyl (DMEQ)



4[4-(6-methoxy-2-benzoxazoyl)-phenyl]



3-(1-pyrenyl) propyl

Figure 6 HPLC of steroids with fluorescent detection – some examples of pre-column derivatives.

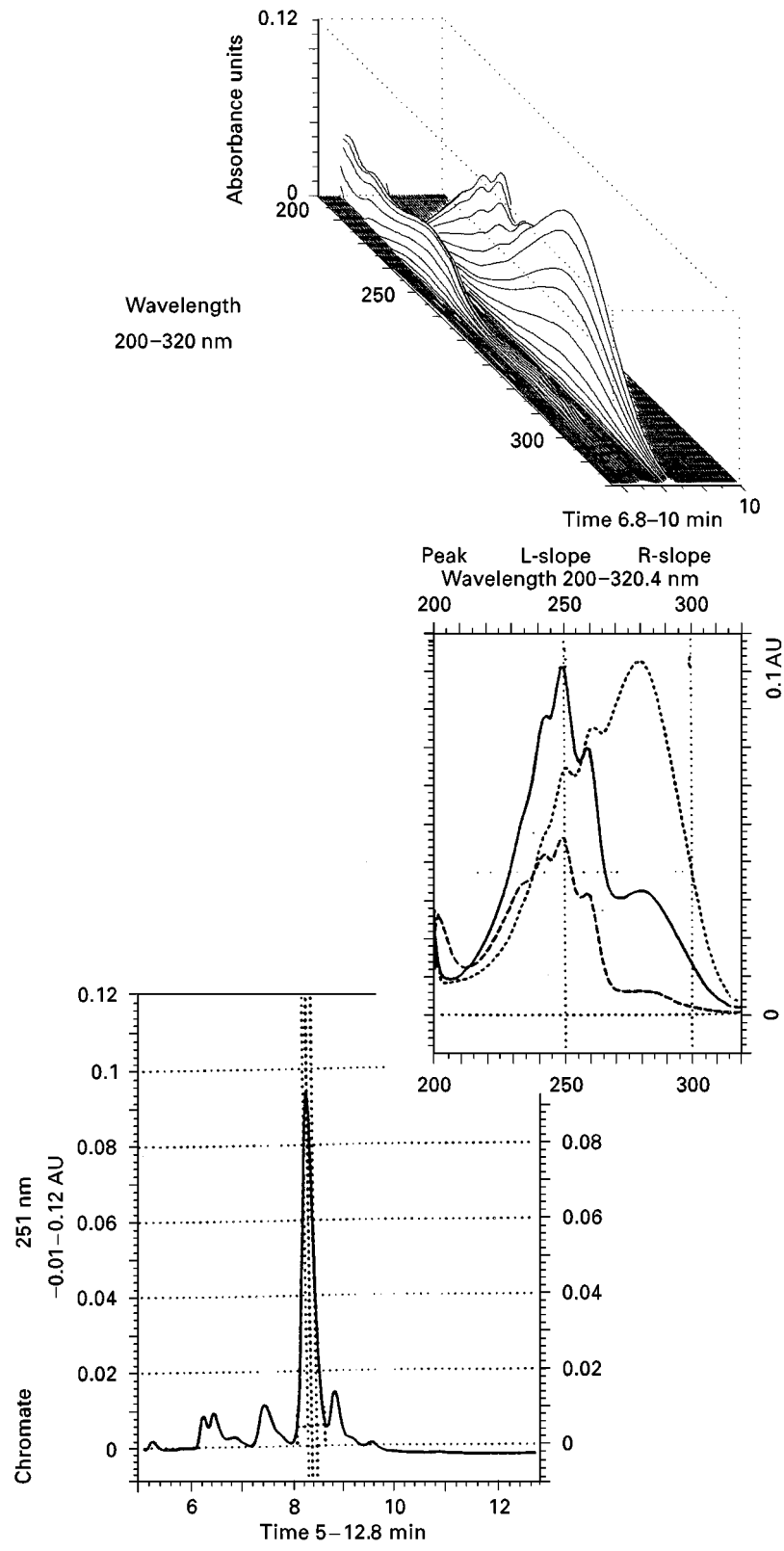


Figure 7 Use of photodiode array detection in HPLC separation of metabolites of 25-hydroxydihydrotachysterol$_3$. Monitoring the eluent at 251 nm indicates what appears to be a single homogeneous peak. Examination of the UV spectra at the leading edge, the apex and the trailing edge clearly demonstrates the presence of a contaminant. From Makin HLJ, Gower DB and Kirk DN (eds) *Steroid Analysis* (1995). With permission from Kluwer Academic Publishers.

Other detection methods can be used, such as UV absorption with or without derivatization, fluorimetry after chemical modification or derivatization or electrochemical detection. These detection/ quantitation methods can usually be carried out online – that is to say, that the HPLC eluent can be directly and continuously monitored. When chemical derivatization is required, this can be carried out prior to HPLC (pre-column derivatization) but this may reduce resolution. In such cases it is also possible to carry out the derivatization after the HPLC separation (post-column derivatization). Some examples of pre-column derivatization used for fluorescent detection are illustrated in **Figure 6**.

One particular method of UV monitoring of eluents from HPLC separations is the photodiode array detector. With this detector the absorption of the eluent is continuously monitored over a range of wavelengths and the data stored in a computer. Reconstructed chromatograms can be obtained at a later date, as can three-dimensional reconstructions (showing time versus absorbance versus wavelength). An example of photodiode array detection is illustrated in **Figure 7**, which demonstrates that what appears to be a single peak when monitored at 251 nm is in fact composed of two unresolved compounds and this can be demonstrated by comparing the UV spectra obtained at the leading edge, the apex and the trailing edge of the peak. This lack of resolution is also seen in the three-dimensional picture. This particular separation was obtained when examining the metabolites of a chemical analogue of 25-hydroxyvitamin D.

HPLC can also be linked to mass spectrometry and, increasingly, techniques are becoming available for the direct linking of the column to the mass spectrometer. In the past, mass spectrometry of HPLC eluents had, like immunoassay, required that eluents be collected and prepared for mass spectrometry. The availability of ionization techniques (such as atmospheric pressure ionization, electrospray, etc.) now allow the HPLC eluent to go directly to the mass spectrometer. Many steroids are susceptible to ionization in such systems but others require derivatization to achieve satisfactory ionization. In these systems the elution solvents should contain water or salts, which implies the use of reversed-phase separation. This is not a particular problem with most steroids but for particular steroid groups (e.g. metabolites of vitamin D) it may require the development of new solvent systems. Such ionization procedures are inevitably low energy and fragmentation is limited. The advantage of this low energy ionization is that intact steroid conjugates can be examined. However, low energy ionization is inefficient, limits the sensitivity of detection and does not yield information about structure. To some degree this structural limitation can be resolved by utilizing LC-MS-MS where the first mass spectrometer allows only the major ion obtained to pass through to a collision cell. Here the ion is subjected to higher energy ionization or bombardment, producing further fragments which can then be analysed by a second mass spectrometer, yielding structural information. Such systems are expensive but are immensely valuable. Unfortunately, LC-MS and LC-MS-MS are not as sensitive as GC-MS, which has been widely used for the measurement of many steroids present at low concentrations in human body fluids. High resolution GC-MS can of course increase sensitivity of detection even further.

Conclusion

The advent of simpler and cheaper mass spectrometers which allow direct coupling of the LC column has meant that less attention needs to be paid to resolution and the development of solvent systems and column packings to achieve improved resolution is no longer as important. Attention has therefore shifted towards increasing sensitivity by the use of microbore columns with the low flow rates required for maximum ionization in the MS and the use of nanospray ESI. Today excellent mass spectra can be obtained using such systems with femtomole concentrations of analyte. There is considerable scope for further enhancement of sensitivity and selectivity by improved MS design of both hardware and software.

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Acknowledgements

Readers are encouraged to seek further information from the texts quoted below which are fully referenced and allow entry to the extensive research literature on this topic. In preparing this review, I acknowledge the debt I owe to all the researchers in this area whose results I have used but whose work I have not been able to acknowledge directly.

See also: **II/Chromatography: Liquid:** Mechanisms: Normal Phase; Mechanisms: Reversed Phases. **III/Sterols:** Thin-Layer (Planar) Chromatography.

Further Reading

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Supercritical Fluid Chromatography

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Supercritical fluid chromatography (SFC) has been recognized as a powerful separation technique complementing gas chromatography (GC) and high performance liquid chromatography (HPLC). Klesper *et al*. published results of the use of supercritical dichlorodifluoromethane and monochlorodifluoromethane to separate involatile nickel porphyrin in 1961. The development of the technique was limited by instrumental and experimental difficulties due to the high temperatures and pressures required to maintain the mobile phase in a supercritical state. Novotny and Lee, however, developed SFC with a capillary column (cSFC) in 1981, which led to significant advances in the technique. In 1982 Gere *et al*. developed an instrument for packed-column SFC (pSFC) based on modification of an HPLC. They demonstrated, using polycyclic aromatic hydrocarbons as probe molecules, that the resolution per unit time in pSFC was 5–10 times better than in HPLC with the same columns, due to more favourable diffusivity in supercritical fluids.

The advantages of SFC have been described elsewhere in this *Encyclopedia*. Pure carbon dioxide fluid is a solvent of inadequate polarity. For the analysis of polar compounds by SFC, alcohol is generally added to a mobile-phase fluid as a modifier. Small amounts of polar modifiers significantly increase the solvent strength of the mobile phase and make it possible to elute polar compounds. In particular, pSFC has been applied to various kinds of polar compounds such as drugs, and shown to be superior to HPLC with respect to analysis time, efficiency and selectivity.

The nonpolar steroids cholesterol and ketosteroids are easily eluted by either cSFC or pSFC with pure carbon dioxide. Synthetic corticosteroids, which are widely used therapeutically for the suppression of adrenocortical functions, inflammatory and allergic diseases, have multiple hydroxyl functional groups in the structures. In order to modify the efficacy and suppress adverse reactions, many corticosteroids have been synthesized. Thin-layer, normal and reversed-phase chromatography have been used for the analysis of these compounds. For a number of the synthetic corticosteroids used in therapy, very little work has been carried out by pSFC. These polar steroids are probably difficult to elute with pure carbon dioxide due to its poor solvent strength.

In this article, the pSFC retention behaviour of synthetic corticosteroids, possessing one to four hydroxyl groups, are focused on. The effect of several parameters (stationary phase, modifiers, pressure and temperature) on retention and efficiency are considered. The chemical structures of corticosteroids are shown in **Figure 1**. They were chromatographed using a pSFC instrument modified from a commercial HPLC system. The addition of methanol to carbon dioxide and adoption of an aminopropyl stationary phase produced both good resolution and symmetric peak shapes. Both plate number and resolution indicated that the maxima were around the critical temperature (40–50°C) of the binary fluid. The selectivity and separation of the analytes in pSFC are superior to those in existing normal and reversed-phase HPLC. Seven polar corticosteroids, possessing one to four hydroxyl groups, showed baseline separation within 6.5 min with a modifier gradient method.

Instrumentation of pSFC

Most studies have been done using commercial pSFC instruments. However, a pSFC with the same performance as a commercial instrument can easily be constructed. The HPLC for pSFC operation requires some simple adaptations to allow use of supercritical carbon dioxide as a mobile phase. The pSFC system constructed by the authors by modifying a Shimadzu HPLC is shown in **Figure 2**.